ubiquinone/ubiquinol exchange between the RC and cytochrome b/c_1 (*12*, *17–20*). Biochemical studies on these systems have shown that PufX is present in the RC-LH1 complex in a 1:1 stoichiometry with the RC and that it has a strong tendency to interact with the LH1 α-polypeptide (*17*). There is evidence that PufX is involved directly in the supramolecular organization of the photosynthetic system, prevents LH1 from completely encircling the RC, and perhaps induces a specific orientation of the RC inside the LH1 complex (*29*).

An equivalent gene for the PufX protein has not been identified in the *Rps. palustris* genome. This is not unexpected, because the PufX protein sequences, even for two such closely related species as *Rb. sphaeroides* and *Rb. capsulatus*, show only 23% identity (*18*); even for the α-helical membrane-spanning core region of PufX sequences, the identity is only 38%. In order to identify helix W, we performed matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry analysis on purified RC-LH1 core complexes. In addition to the expected α- and β-apoproteins and the L, M, and H subunits of the RC, this revealed the presence of a protein of mass 10,707 D. So far, this protein appears to be N-terminally blocked and has resisted attempts to sequence it. There are more than 10 candidate genes in the *Rps. palustris* genome that could encode for putative membrane proteins of this mass. Efforts to identify the W gene are continuing.

References and Notes

1. R. van Grondelle, J. P. Dekker, T. Gillbro, V. Sundstrom, *Biochim. Biophys. Acta Bioenergetics* **1187**, 1 (1994).
2. R. J. Cogdell *et al.*, *J. Bacteriol.* **181**, 3869 (1999).
3. J. Deisenhofer, O. Epp, K. Miki, R. Huber, H. Michel, *Nature* **318**, 618 (1985).
4. G. Fritzsch, J. Koepke, R. Diem, A. Kuglstatter, L. Baciou, *Acta Crystallogr.* **D58**, 1660 (2002).
5. G. McDermott *et al.*, *Nature* **374**, 517 (1995).
6. M. Z. Papiz, S. M. Prince, T. Howard, R. J. Cogdell, N. W. Isaacs, *J. Mol. Biol.* **326**, 1523 (2003).
7. J. P. Abrahams, A. G. W. Leslie, R. Lutter, J. E. Walker, *Nature* **370**, 621 (1994).
8. S. Iwata *et al.*, *Science* **281**, 64 (1998).
9. S. Karrasch, P. A. Bullough, R. Ghosh, *EMBO J.* **14**, 631 (1995).
10. S. J. Jamieson *et al.*, *EMBO J.* **21**, 3927 (2002).
11. T. Walz, S. J. Jamieson, C. M. Bowers, P. A. Bullough, C. N. Hunter, *J. Mol. Biol.*, **282**, 833 (1998).
12. T. Walz, R. Ghosh, *J. Mol. Biol.* **265**, 107 (1997).
13. A. Gall, thesis, University of Glasgow, UK (1994).
14. C. Jungas, J. L. Ranck, J. L. Rigaud, P. Joliot, A. Vermeglio, *EMBO J.* **18**, 534 (1999).
15. C. C. Moser *et al.*, *Adv. Protein Chem.* **63**, 71 (2003).
16. R. J. Cogdell *et al.*, *Photosynth. Res.* **48**, 55 (1996).
17. P. A. Recchia *et al.*, *Biochemistry* **37**, 11055 (1998).
18. P. S. Parkes-Loach *et al.*, *Biochemistry* **40**, 5593 (2001).
19. W. P. Barz *et al.*, *Biochemistry* **34**, 15235 (1995).
20. W. P. Barz *et al.*, *Biochemistry* **34**, 15248 (1995).
21. T. G. Lilburn, C. E. Haith, R. C. Prince, J. T. Beatty, *Biochim. Biophys. Acta* **1100**, 160 (1992).
22. P. McGlynn, C. N. Hunter, M. R. Jones, *FEBS Lett.* **349**, 349 (1994).
23. P. McGlynn, W. H. J. Westerhuis, M. R. Jones, C. N. Hunter, *J. Biol. Chem.* **271**, 3285 (1996).
24. Materials and methods are available as supporting material on *Science* Online.
25. K. E. McAuley *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 14706 (1999).
26. M. H. B. Stowell *et al.*, *Science* **276**, 812 (1997).
27. K. E. McAuley *et al.*, *Biochemistry* **39**, 15032 (2000).
28. S. Scheuring *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 1690 (2003).
29. R. N. Frese *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 5197 (2000).
30. M. Carson, *Methods Enzymol.* **277**, 493 (1997).

31. We thank A. Pitt for the mass spectrometry analyses and J. T Beatty for bioinformatics. Coordinates have been deposited with the Protein Data Bank (accession code 1PYH). Supported by grants from the Wellcome Trust and the Biotechnology and Biological Sciences Research Council.

Supporting Online Material

www.sciencemag.org/cgi/content/full/302/5652/1969/DC1
Materials and Methods
Figs. S1 to S5

7 July 2003; accepted 20 October 2003

Mono- Versus Polyubiquitination: Differential Control of p53 Fate by Mdm2

Muyang Li, Christopher L. Brooks, Foon Wu-Baer, Delin Chen, Richard Baer, Wei Gu*

Although Mdm2-mediated ubiquitination is essential for both degradation and nuclear export of p53, the molecular basis for the differential effects of Mdm2 remains unknown. Here we show that low levels of Mdm2 activity induce monoubiquitination and nuclear export of p53, whereas high levels promote p53's polyubiquitination and nuclear degradation. A p53-ubiquitin fusion protein that mimics monoubiquitinated p53 was found to accumulate in the cytoplasm in an Mdm2-independent manner, indicating that monoubiquitination is critical for p53 trafficking. These results clarify the nature of ubiquitination-mediated p53 regulation and suggest that distinct mechanisms regulate p53 function in accordance with the levels of Mdm2 activity.

The p53 tumor suppressor protein induces cell growth arrest, apoptosis, and senescence in response to various types of stress (*1*). In unstressed cells, p53 is maintained at low levels by the action of Mdm2, an oncogenic E3 ligase. Numerous studies indicate that the ubiquitin ligase activity of Mdm2 is essential for both degradation and nuclear export of p53 (*2–12*). We investigated the molecular basis for the differential effects of Mdm2 on p53 fate.

To examine whether Mdm2 alone catalyzes polyubiquitination (conjugation with a polymeric ubiquitin chain) or only monoubiquitination (conjugation with a ubiquitin monomer at one or multiple sites) of p53, we performed an in vitro ubiquitination assay using purified components (fig. S1A). Incubation of Flag-p53 with glutathione *S*-transferase (GST)–Mdm2 in the presence of E1, E2, and ubiquitin generated ubiquitin-conjugated forms of p53 (fig. S1B). We then tested whether Mdm2 induced the same effect with a mutant form of ubiquitin (UbK0), in which all seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) were replaced by arginine (fig. S1C). Because this mutant lacks potential sites for polyubiquitination, it should support only monoubiquitination. The patterns of p53-ubiquitin conjugates generated by wild-type and mutant ubiquitin were indistinguishable (fig. S1B), indicating that Mdm2 primarily catalyzes monoubiquitination of p53 at multiple sites under these conditions.

Yet, in contrast to these results, we and others have previously demonstrated that Mdm2 alone is apparently sufficient to induce polyubiquitination of p53 when in vitro–translated p53 polypeptides are used as substrates for in vitro ubiquitination (*11*, *13–17*). To test the possibility that the outcome (mono versus poly) of Mdm2-mediated ubiquitination is influenced by the enzyme: substrate ratio, we prepared ubiquitination reactions containing a constant amount of recombinant p53 and varying amounts of Mdm2 (Fig. 1A). Monoubiquitination of p53 was observed when the Mdm2:p53 ratio was low, whereas slower-migrating, polyubiquitinated forms of p53 were observed when the Mdm2:p53 ratio reached 3.6 or higher. Because the polyubiquitination-defective ubiquitin mutant (UbK0) only supported the faster-migrating, monoubiquitinated forms of p53 (Fig. 1B), the higher-molecular-weight ubiquitin conjugates clearly represent polyubiquitinated p53. These data indicate that Mdm2 catalyzes both mono- and polyubiquitination in a dosage-dependent manner.

Institute for Cancer Genetics and Department of Pathology, College of Physicians & Surgeons, Columbia University, 1150 St. Nicholas Avenue, New York, NY 10032, USA.

*To whom correspondence should be addressed. E-mail: wg8@columbia.edu

We next tested whether low levels of Mdm2 activity can induce monoubiquitination of p53 in vivo. To detect ubiquitinated forms of cellular p53, we transfected H1299 cells (a p53-null human lung carcinoma cell line) with expression vectors encoding His_6-tagged ubiquitin, p53, and Mdm2, and then isolated ubiquitinated polypeptides by nitrilotriacetic acid (NTA) affinity chromatography (*18*, *19*). Western blot analysis of the affinity-purified ubiquitin conjugates with a p53 antibody (DO-1) indicated that low levels of Mdm2 activity primarily induced monoubiquitination of p53 (Fig. 1C) without dramatically affecting its stability (Fig. 1D). In similar assays with the ubiquitin mutant UbK0, Mdm2 expression generated the same pattern of monoubiquitinated p53 conjugates (Fig. 1C). To exclude the possibility that the levels of ubiquitin (Ub or UbK0) have an effect on the outcome of p53 ubiquitination, we also transfected cells with varying amounts of Ub and UbK0 and obtained the same results (*20*). To examine the function of p53 monoubiquitination, we transfected H1299 cells with a green fluorescent protein (GFP)–tagged derivative of p53 (GFP-p53) alone or together with a low level of Mdm2 (*19*). In the absence of Mdm2, GFP-p53 showed a predominantly nuclear pattern of localization (fig. S2A). In contrast, low levels of Mdm2 induced cytoplasmic translocation of GFP-p53 (fig. S2A). Western blot analysis of subcellular fractions confirmed that monoubiquitinated p53 was located predominantly in the cytoplasm (Fig. 1E). These results suggest that low Mdm2 levels induce both monoubiquitination and cytoplasmic translocation of p53.

To investigate whether monoubiquitination is sufficient for cytoplasmic translocation of p53, we designed a molecule that mimics the monoubiquitinated form of p53. It was previously reported that direct fusion of ubiquitin sequences to the yeast α-factor receptor causes its subcellular redistribution in a manner akin to posttranslational monoubiquitination (*21*). Thus, we constructed a p53 derivative (p53-Ub) in which one copy of the ubiquitin sequence was fused to the C terminus of wild-type p53 (wt p53) (Fig. 2A) and then transfected H1299 cells with expression vectors encoding either wt p53 or the p53-Ub fusion protein. As expected, ectopic expression of wt p53 yielded nuclear staining in more than 75% of cells. The p53-Ub fusion protein showed predominantly cytoplasmic localization (Fig. 2B), whereas in-frame fusion of the ubiquitin sequence to the Max transcription factor, a nuclear protein known not to be regulated by monoubiquitination, had no effect on its subcellular distribution (Fig. 2C). These results were confirmed by Western blot analysis of subcellular fractions (fig. S3A). Similar immunostaining patterns were also observed in Mdm2/p53-DKO mouse embryonic fibroblasts (Fig. 2B), implying that monoubiquitinated p53 can be translocated to the cytoplasm without Mdm2. These results provide evidence that Mdm2 promotes the cytoplasmic translocation of p53 by catalyzing its ubiquitination, not by physically escorting p53 into the cytoplasm.

To confirm that Mdm2 can induce p53 polyubiquitination in vivo, we transfected H1299 cells with varying amounts of Mdm2. As expected, low levels of Mdm2 mainly induced monoubiquitination of p53; however, significant amounts of slower-migrating,

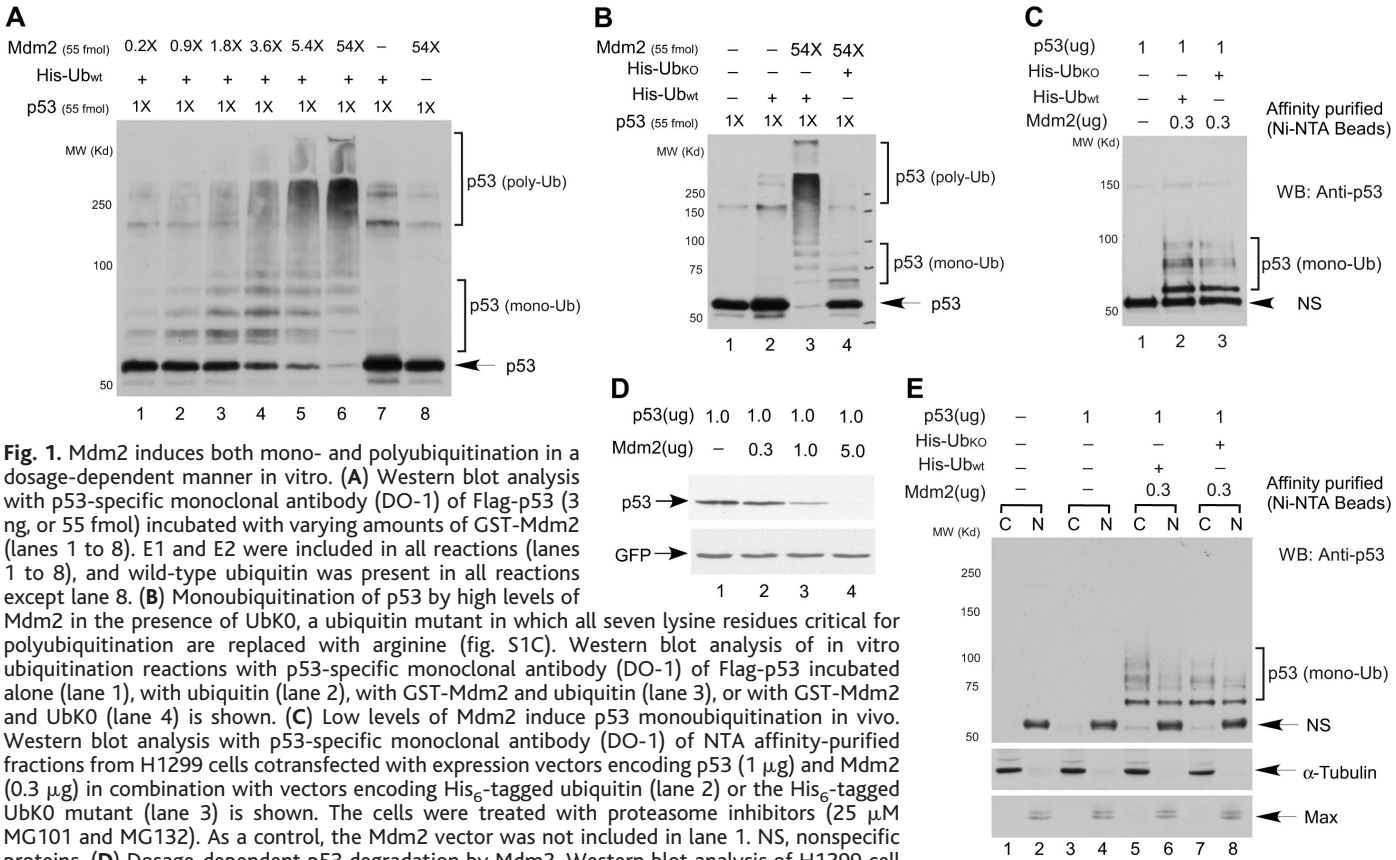

Fig. 1. Mdm2 induces both mono- and polyubiquitination in a dosage-dependent manner in vitro. (**A**) Western blot analysis with p53-specific monoclonal antibody (DO-1) of Flag-p53 (3 ng, or 55 fmol) incubated with varying amounts of GST-Mdm2 (lanes 1 to 8). E1 and E2 were included in all reactions (lanes 1 to 8), and wild-type ubiquitin was present in all reactions except lane 8. (**B**) Monoubiquitination of p53 by high levels of Mdm2 in the presence of UbK0, a ubiquitin mutant in which all seven lysine residues critical for polyubiquitination are replaced with arginine (fig. S1C). Western blot analysis of in vitro ubiquitination reactions with p53-specific monoclonal antibody (DO-1) of Flag-p53 incubated alone (lane 1), with ubiquitin (lane 2), with GST-Mdm2 and ubiquitin (lane 3), or with GST-Mdm2 and UbK0 (lane 4) is shown. (**C**) Low levels of Mdm2 induce p53 monoubiquitination in vivo. Western blot analysis with p53-specific monoclonal antibody (DO-1) of NTA affinity-purified fractions from H1299 cells cotransfected with expression vectors encoding p53 (1 μg) and Mdm2 (0.3 μg) in combination with vectors encoding His_6-tagged ubiquitin (lane 2) or the His_6-tagged UbK0 mutant (lane 3) is shown. The cells were treated with proteasome inhibitors (25 μM MG101 and MG132). As a control, the Mdm2 vector was not included in lane 1. NS, nonspecific proteins. (**D**) Dosage-dependent p53 degradation by Mdm2. Western blot analysis of H1299 cell extracts cotransfected with CMV-p53 (1 μg) and CMV-GFP (1 μg) (lane 1), together with varying amounts of CMV-Mdm2 (0 to 5 μg) (lanes 1 to 4) using p53-specific (upper) and GFP-specific antibodies (lower) is shown. (**E**) Cytoplasmic translocation of monoubiquitinated p53. Shown is Western blot analysis with p53-specific monoclonal antibody (DO-1) of NTA affinity-purified fractions from different subcellular compartments (C, cytoplasm; N, nuclei) of H1299 cells cotransfected with expression vectors encoding p53 (1 μg) and Mdm2 (0.3 μg) in combination with vectors encoding His-ubiquitin (lanes 5 and 6), or the His-UbK0 mutant (lanes 7 and 8). The cells were treated with proteasome inhibitors (25 μM MG101 and MG132). A cytoplasmic marker protein (α-tubulin) and a nuclear transcription factor (Max) were used as controls to confirm the quality of the cytoplasmic and nuclear fractions.

polyubiquitinated p53 were generated under increasing levels of Mdm2 expression in the presence of wild-type ubiquitin but not the UbK0 mutant (Fig. 3A). Western blot analysis of immunoprecipitated p53 with an antibody (FK1) that specifically recognizes polyubiquitin chains confirmed a dosage-dependent induction of polyubiquitinated p53 (fig. S4A). Again, the levels of polyubiquitinated p53 were significantly reduced in the presence of UbK0 (fig. S4A). Thus, p53 is polyubiquitinated in vivo in the presence of high levels of Mdm2 activity.

We also tested the effect of polyubiquitination on subcellular localization of p53 (Fig. 3B). When H1299 cells were transfected with GFP-tagged p53, the GFP-p53 fusion protein was readily detected in the nucleus; however, when the same cells were cotransfected with both GFP-p53 and high levels of Mdm2, GFP-p53 was almost undetectable, suggesting that GFP-p53 is degraded in an Mdm2-dependent manner. To visualize the site of Mdm2-mediated p53 degradation, we treated these cells with two proteasome inhibitors (MG101 and MG132) before fixation. Surprisingly, GFP-p53 staining was primarily detected in the nucleus after treatment (Fig. 3B). Because proteasome inhibitors had no effect on the subcellular distribution of p53 (fig. S2A) (*11*), these data suggest that, in contrast to monoubiquitination, Mdm2-dependent polyubiquitination does not promote cytoplasmic translocation of p53. Western blot analysis of different subcellular fractions confirmed that polyubiquitinated p53 is mainly present in the nuclei under these conditions (fig. S4B). Thus, high levels of Mdm2 induce both polyubiquitination and nuclear degradation of p53.

It is widely accepted that Mdm2 is a key mediator of p53 degradation and that the endogenous levels of Mdm2 are dynamically regulated through the p53-Mdm2 feedback loop (*1*, *22*). Several investigators have proposed that p53 is translocated to, and degraded in, the cytoplasm, on the basis of the fact that p53 can be stabilized by blocking its nuclear export (*9*, *10*). However, this proposal seems at odds with other reports that p53 degradation can also occur in the nucleus (*18*, *23*, *24*), presumably by nuclear proteasomes. Our findings suggest that these seemingly contradictory observations may reflect differential activities of Mdm2 that are dictated by its intracellular concentration. Because Mdm2 is maintained at low levels in un-

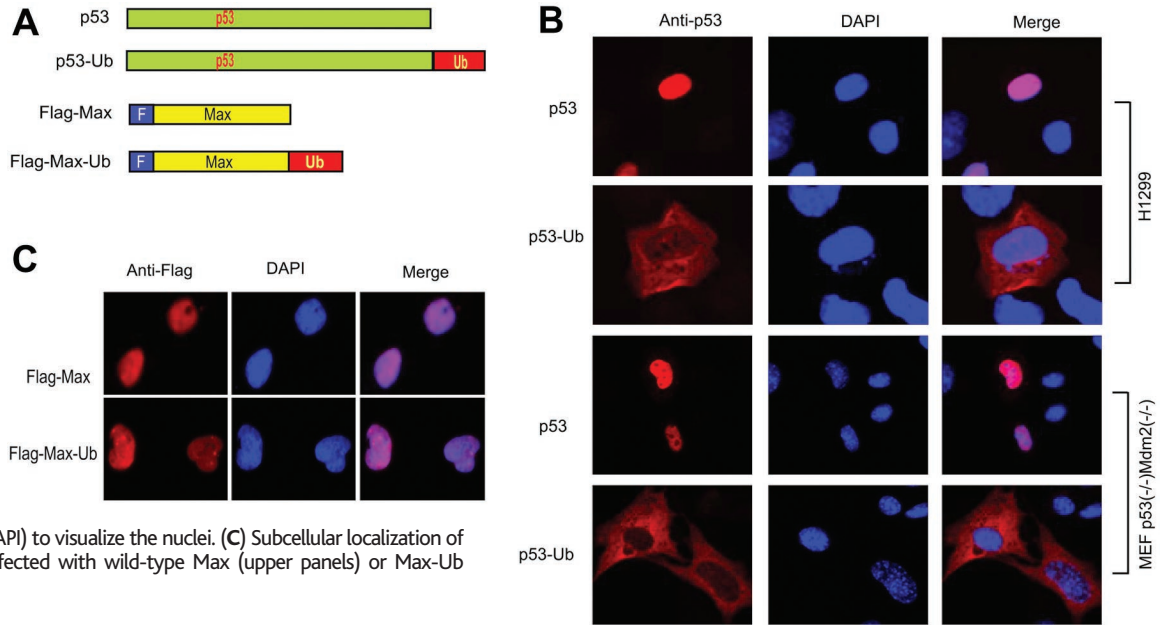

Fig. 2. A p53-ubiquitin fusion protein that mimics monoubiquitinated p53 accumulates predominantly in the cytoplasm. (**A**) Schematic representation of wt p53 and Max polypeptides along with their derivatives (p53-Ub and Max-Ub) that have one copy of the ubiquitin sequence fused to the C terminus. (**B**) Subcellular localization of p53 in H1299 cells and p53/Mdm2-DKO mouse embryonic fibroblasts transfected with wt p53 or p53-Ub. Cells were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) to visualize the nuclei. (**C**) Subcellular localization of Max in H1299 cells transfected with wild-type Max (upper panels) or Max-Ub (lower panels).

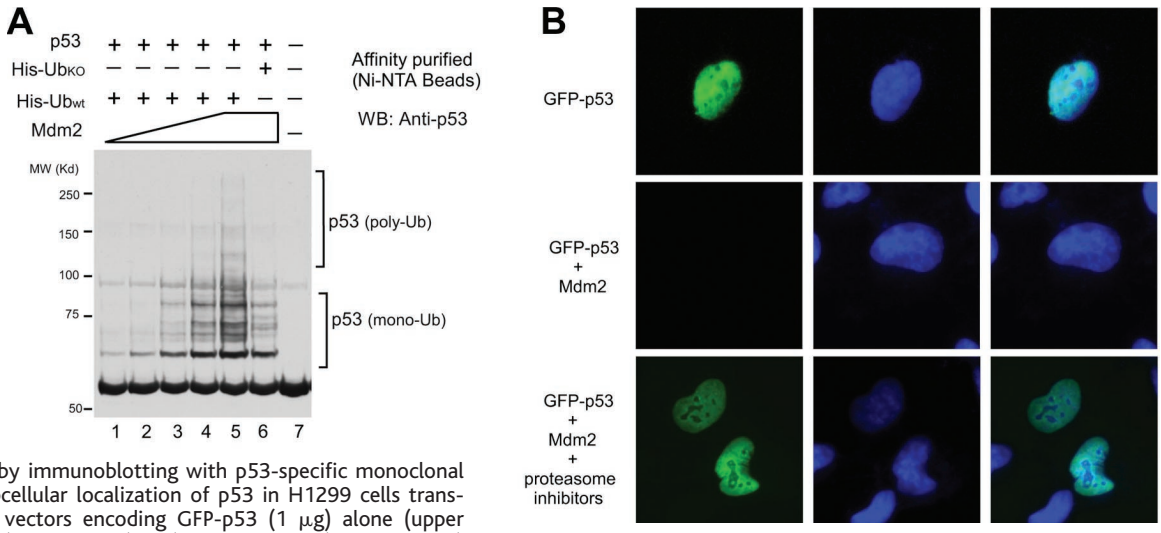

Fig. 3. High levels of Mdm2 activity induce polyubiquitination and nuclear degradation of p53 in vivo. (**A**) H1299 cells were cotransfected with expression vectors encoding p53 (1 μg) and Mdm2 (0.1 to 5 μg) in combination with vectors encoding His-ubiquitin (lanes 1 to 5) or the His-UbK0 mutant (lane 6). The ubiquitinated polypeptides were purified from cell lysates by NTA affinity chromatography and analyzed by immunoblotting with p53-specific monoclonal antibody DO-1. (**B**) Subcellular localization of p53 in H1299 cells transfected with expression vectors encoding GFP-p53 (1 μg) alone (upper panels) or GFP-p53 (1 μg) and Mdm2 (7 μg) in the absence (middle panels) or presence (lower panels) of proteasome inhibitors (25 μM MG101 and MG132). Cells were counterstained with DAPI to visualize the nuclei.

stressed cells, it is likely that Mdm2-mediated monoubiquitination and cytoplasmic translocation of p53 play an important role in unstressed cells, and that blocking nuclear export in this setting may stabilize p53 in the nucleus (*9*, *10*). However, when Mdm2 activities are high, Mdm2-mediated polyubiquitination induces p53 degradation in the nucleus. Although we cannot formally exclude the possibility that some polyubiquitinated p53 molecules are exported from the nucleus and are either deubiquitinated or degraded in the cytoplasm, our hypothesis is supported by recent reports that p53 is degraded in the nucleus under conditions of Mdm2 overexpression and during late stages of the DNA damage response (*18*, *23*, *24*). It is also interesting to consider that the E3 ligase activity of endogenous Mdm2 may be modulated in vivo by posttranslational modifications (*25*, *26*) or recruitment of other cofactors (*27*, *28*).

The physiological role of p53 monoubiquitination is still an open question. Given that the multistep process of polyubiquitination is both time- and energy-consuming (*29*), we propose that monoubiquitination and the resulting cytoplasmic translocation of p53 provide a rapid but reversible mechanism for down-regulating p53 function. Further studies are needed to investigate how monoubiquitinated p53 is further processed (either degraded or deubiquitinated). It is possible that the HAUSP ubiquitin hydrolase, in addition to stabilizing p53 (*30*), also regulates its cytoplasmic translocation by deubiquitinating monoubiquitinated p53. It is also very likely that additional cellular factors are necessary to facilitate p53 degradation (*27*, *28*, *31*, *32*), particularly when endogenous Mdm2 activities are not sufficient to catalyze p53 polyubiquitination directly.

References and Notes

1. B. Vogelstein, D. Lane, A. J. Levine, *Nature* **408**, 307 (2000).
2. R. Honda, H. Tanaka, H. Yasuda, *FEBS Lett.* **420**, 25 (1997).
3. Y. Haupt, R. Maya, A. Kazaz, M. Oren, *Nature* **387**, 296 (1997).
4. M. H. Kubbutat, S. N. Jones, K. H. Vousden, *Nature* **387**, 299 (1997).
5. S. Fang, J. P. Jensen, R. L. Ludwig, K. H. Vousden, A. M. Weissman, *J. Biol. Chem.* **275**, 8945 (2000).
6. Z. Lai *et al.*, *J. Biol. Chem.* **276**, 31357 (2001).
7. S. D. Boyd, K. Y. Tsai, T. Jacks, *Nature Cell Biol.* **2**, 563 (2000).
8. R. K. Geyer, Z. K. Yu, C. G. Maki, *Nature Cell Biol.* **2**, 569 (2000).
9. D. A. Freedman, A. J. Levine, *Mol. Cell Biol.* **18**, 7288 (1998).
10. J. M. Stommel *et al.*, *EMBO J.* **18**, 1660 (1999).
11. M. A. Lohrum, D. B. Woods, R. L. Ludwig, E. Balint, K. H. Vousden, *Mol. Cell Biol.* **21**, 8521 (2001).
12. J. Gu, L. Nie, D. Wiederschain, Z. M. Yuan, *Mol. Cell Biol.* **21**, 8533 (2001).
13. M. Li, J. Luo, C. L. Brooks, W. Gu, *J. Biol. Chem.* **277**, 50607 (2002).
14. M. S. Rodriguez, J. M. Desterro, S. Lain, D. P. Lane, R. T. Hay, *Mol. Cell Biol.* **20**, 8458 (2000).
15. S. Nakamura, J. A. Roth, T. Mukhopadhyay, *Mol. Cell Biol.* **20**, 9391 (2000).
16. C. A. Midgley *et al.*, *Oncogene* **19**, 2312 (2000).
17. S. Y. Fuchs, V. Adler, T. Buschmann, X. Wu, Z. Ronai, *Oncogene* **17**, 2543 (1998).
18. D. P. Xirodimas, C. W. Stephen, D. P. Lane, *Exp. Cell Res.* **270**, 66 (2001).
19. Materials and methods are available as supporting material on *Science* Online.
20. M. Li, C. L. Brooks, W. Gu, unpublished results.
21. J. Terrell, S. Shih, R. Dunn, L. Hicke, *Mol. Cell* **1**, 193 (1998).
22. R. Lev Bar-Or *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 11250 (2000).
23. T. R. Shirangi, A. Zaika, U. M. Moll, *FASEB J.* **16**, 420 (2002).
24. Z. K. Yu, R. K. Geyer, C. G. Maki, *Oncogene* **19**, 5892 (2000).
25. C. Blattner, T. Hay, D. W. Meek, D. P. Lane, *Mol. Cell Biol.* **22**, 6170 (2002).
26. K. Okamoto *et al.*, *Mol. Cell* **9**, 761 (2002).
27. J. M. Parant, G. Lozano, *Hum. Mutat.* **21**, 321 (2003).
28. S. R. Grossman *et al.*, *Science* **300**, 342 (2003).
29. C. M. Pickart, *Annu. Rev. Biochem.* **70**, 503 (2001).
30. M. Li *et al.*, *Nature* **416**, 648 (2002).
31. R. P. Leng *et al.*, *Cell* **112**, 779 (2003).
32. A. Y. Nikolaev, M. Li, N. Puskas, J. Qin, W. Gu, *Cell* **112**, 29 (2003).

33. We thank Z.-J. Chen for constructs and helpful discussions and Y.-P. Zhang for sharing unpublished data and critical suggestions. Supported by grants from the Avon Foundation, the Irma T. Hirschl Trust, and the National Institutes of Health/National Cancer Institute to W.G. and R.B. W.G. is a Leukemia & Lymphoma Society Scholar.

Supporting Online Material

www.sciencemag.org/cgi/content/full/302/5652/1972/DC1
Materials and Methods
Figs. S1 to S4
References

10 September 2003; accepted 28 October 2003

Redistribution of Intracellular Oxygen in Hypoxia by Nitric Oxide: Effect on HIF1α

Thilo Hagen,[1] Cormac T. Taylor,[2] Francis Lam,[1] Salvador Moncada[1]*

Cells exposed to low oxygen concentrations respond by initiating defense mechanisms, including the stabilization of hypoxia-inducible factor (HIF) 1α, a transcription factor that upregulates genes such as those involved in glycolysis and angiogenesis. Nitric oxide and other inhibitors of mitochondrial respiration prevent the stabilization of HIF1α during hypoxia. In studies of cultured cells, we show that this effect is a result of an increase in prolyl hydroxylase–dependent degradation of HIF1α. With the use of Renilla luciferase to detect intracellular oxygen concentrations, we also demonstrate that, upon inhibition of mitochondrial respiration in hypoxia, oxygen is redistributed toward non-respiratory oxygen-dependent targets such as prolyl hydroxylases so that they do not register hypoxia. Thus, the signaling consequences of hypoxia may be profoundly modified by nitric oxide.

HIF plays a major role in the response of tissues to low partial pressures of O_2 (*1*). The protein stability of the α subunit (HIF1α) of this heterodimeric transcription factor is regulated in an O_2-dependent manner (*2*–*4*) by a family of prolyl hydroxylases (*5*, *6*). At low O_2 concentrations, prolyl hydroxylase activity is inhibited, and HIF1α accumulates to heterodimerize with HIF1β and activate the expression of HIF-dependent target genes. In earlier work, we found that inhibition of mitochondrial respiration by low concentrations (< 100 μM) of nitric oxide (NO), the endogenous inhibitor of cytochrome c oxidase (complex IV of the respiratory chain), leads to inhibition of HIF1α stabilization at a low O_2 concentration (3%). This effect is mimicked by other inhibitors of the respiratory chain, irrespective of the complex at which they act (*7*).

To explore the underlying mechanism, we investigated the effect of various mitochondrial respiratory inhibitors, including NO, on HEK293 cells (a human embryonic kidney cell line) grown under hypoxic conditions (1% O_2). Consistent with our previous results, this treatment prevented the accumulation and transcriptional activity of HIF1α (Fig. 1) (*8*). Inhibition of mitochondrial respiration also prevented HIF1α stabilization in a number of other cell lines (Fig. 1C). We next determined whether this effect was a result of decreased synthesis or increased degradation of HIF1α protein. Hypoxia-dependent HIF1α stabilization was measured in the presence of the proteasome inhibitor MG-132, which inhibits the degradation of ubiquitinated HIF1α (Fig. 2A). At 21% O_2, ubiquitinated HIF1α accumulated in the presence of MG-132. At 1% O_2, the respiratory inhibitor myxothiazol pre-

[1]Wolfson Institute for Biomedical Research, University College London, Gower Street, London WC1E 6BT, UK. [2]Conway Institute for Biomedical and Biochemical Research, University College Dublin 4, Belfield, Dublin, Ireland.

*To whom correspondence should be addressed. E-mail: s.moncada@ucl.ac.uk